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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/10, C12Q 1/02

A1

(11) International Publication Number: **WO 99/06542**

(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/GB98/02269

(22) International Filing Date: 29 July 1998 (29.07.98)

(30) Priority Data:
9716094.9 30 July 1997 (30.07.97) GB

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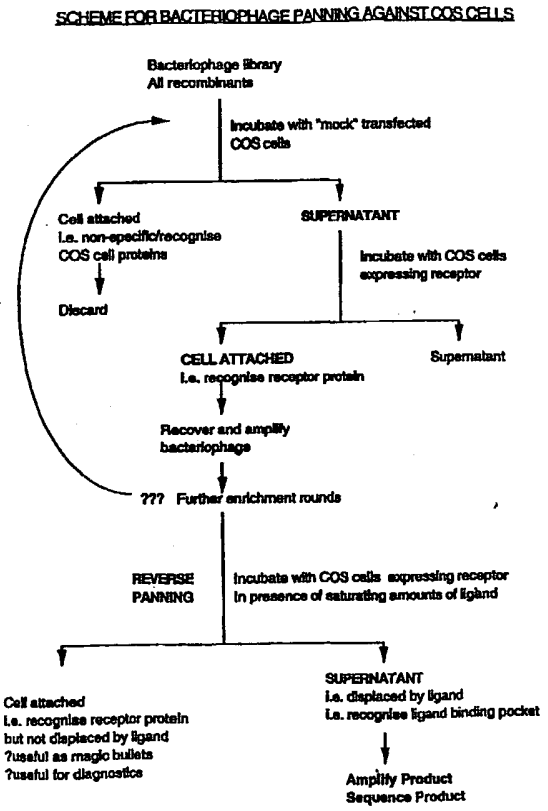
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GM, HR, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR SCREENING BACTERIOPHAGE LIBRARIES

(57) Abstract

A method is provided for producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a heterologous (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected. The process comprises: (a) subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to one or more proteins other than said heterologous protein are bound to the cells; (b) separating bound and unbound bacteriophage; and (c) isolating a modified bacteriophage library depleted in library members which bind to one or more proteins other than said heterologous protein. The modified bacteriophage libraries produced by the method, peptide ligands identified and/or isolated by screening procedures using the modified libraries and the use of identified ligands and consensus sequences thereof in the design of pharmaceutical and diagnostic agents also form part of the invention.



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METHODS FOR SCREENING BACTERIOPHAGE LIBRARIES

This invention relates to methods for screening bacteriophage libraries, to modified bacteriophage libraries for use in such methods, to peptide ligands identified and/or isolated by the described procedures and to the use of identified ligands and consensus sequences thereof in the design of pharmaceutical and diagnostic agents.

The use of so-called bacteriophage libraries for identifying oligonucleotide sequences that are associated with biological ligand/receptor interactions has provided a valuable technique in the quest for novel pharmaceutical substances. Typically, filamentous bacteriophage, such as the filamentous coliphage M13 are genetically modified by adding random oligonucleotides to the coding sequences that encode one of the bacteriophage coat proteins. The coat proteins of the bacteriophage particles are consequently expressed with a random collection of N-terminal oligopeptides. The number of possible oligonucleotides which may be constructed from the 20 naturally occurring amino acids is given by the formula 20^n, where n is the number of amino acids in the oligonucleotide. As will be appreciated, the number of possible oligonucleotides of even a modest size (n=7) is enormous (1.28×10^8), and for larger molecules (n=15) the number of possibilities escalates to 3×10^{19}.

A typical library may contain from 10^6 to 10^{10} recombinant bacteriophage, each of which is distinguished by a coat protein (e.g. gp3 or gp8 in the case of phage M13) bearing a different N-terminal oligopeptide. Where the size of the oligonucleotide is relatively low, e.g. 7-10, every possible oligonucleotide may be represented in the library. Thus, in the case of a bacteriophage library in which the individual members have coat proteins with N-terminal 7-mers, the number of possible different 7-mers (assuming all 20 amino acids are represented) is 20^7, i.e. 1.28×10^8 combinations and each of the possible sequences may be present in the library.

Bacteriophage display of peptide libraries is widely used to select peptides that recognise targets. In a typical use of a bacteriophage library to identify a ligand sequence associated with a particular receptor protein, the protein is immobilised on a support and the immobilised protein "panned" with a suspension of the library of bacteriophage particles. Those bacteriophage having coat proteins with N-terminal sequences which bind to the immobilised receptor protein will be preferentially retained and bacteriophage without recognised N-terminal sequences may be eliminated by washing. The conventional approach is to attach the target to a plastic dish and pan the bacteriophage peptide library against this. Non-adherent bacteriophage are washed from the plate and bacteriophage that adhere to the target are eluted by washing and the selected bacteriophage are amplified in bacteria. The sequence(s) of the recognised N-terminal oligopeptide(s) may then be determined by sequencing the part of the phage DNA that codes for the coat protein.

The above-described technique has proved a very powerful approach to select peptides that recognise targets in this way. Thus, for example, a procedure of this type for constructing and screening peptide libraries is described in WO 91/19818 (Affymax Technologies N.V.)

Libraries are commercially available with 7-mers attached to coat protein gp3 of bacteriophage M13. One such library is the Ph.D.® Phage Display Peptide Library Kit, supplied by New England Biolabs. Inc.

For cell surface-associated proteins including receptors, this conventional approach has not proved effective as receptor proteins frequently possess a tertiary structure that is influenced by interactions between regions of the protein and the phospholipid bilayer or plasma membrane. These interactions often influence the overall folding or conformation of the cell surface protein or receptor. Many receptors and plasma membrane proteins interact with the plasma membrane at multiple sites of the protein and so the overall topography can be influenced by these multiple interactions. Proteins must be folded correctly if they are to act

appropriately and proper folding is essential for receptors to interact with ligands. Membrane proteins can span the membrane and may span the membrane more than once. Examples of these are the transporter proteins, seven transmembrane domain receptors and ion channels. For all these examples, the topography of the protein relative to the plasma membrane is important for the functioning of these proteins.

For conventional panning approaches, the target protein must first be solubilised, isolated and purified. For membrane proteins, the initial solubilisation requires that the plasma membrane is disrupted by the use of detergents. Preparation of the target protein for attachment to plastic dishes may destroy the normal architecture and prevent the protein from adopting the correct conformation.

Previous attempts to pan bacteriophage libraries against membrane proteins in their native state have been problematical. Thus, for cell surface proteins and receptors, it is frequently essential to maintain the protein in the plasma membrane. A number of groups have attempted to pan against the receptor expressed on the native cell but it has been difficult to obtain specific peptide selection using this method.

There are two main problems:

Firstly, the "noise". The native cell expresses many cell surface proteins which will be recognised by peptides and these will be positively selected at the same time as the peptide sequences that recognise the desired target protein. As the desired cell surface protein may be a minor component on the cell surface, the majority of the selected bacteriophage will be possess peptides that recognise proteins that are not the desired target.

Secondly, the "signal". Native cells rarely express a large amount of any one given protein on their cell surface. For instance, a receptor is considered to be

abundant if there are 100,000 copies per cell. Other proteins and receptors may be very rare species. For instance, many growth factor receptors are present at low levels on the cell surface. Therefore, the amount of target is very low.

WO 95/02823 (Brann, M) describes the use of whole cells as substrates for screening peptides, but does not refer to the use of bacteriophage expression libraries.

WO 92/15702 (Chiron Corp.) describes the screening of bacteriophage libraries against whole cells and discusses the problems associated with the use of whole cells as substrate. Thus, lines 11 to 16 on page 6 state that "[p]anning against whole cells is problematical because one tends to select compounds having an affinity for the binding moiety present in the highest concentration (e.g., other surface receptors, carbohydrates, and the like). Further, it appears that some moieties may intrinsically be capable of binding a peptide with higher affinity than other moieties (for example, some receptors may have a deep cleft which permits maximal interaction with a peptide)". The authors of WO 92/15702 seek to mitigate these difficulties by panning against a first substrate which contains the desired target, followed by separating the binding compounds and panning them against an alternate substrate which also contains the desired target. The results presented were disappointing and in Example 4, it was admitted that "no significant increase in phage yield was observed, which indicates that the phage were not enriched by the procedure".

Thirdly, a given peptide which binds specifically to a receptor protein of interest, might additionally bind to another receptor protein, for example one of related structure. The binding to the other protein of related structure might therefore interfere with the binding of the protein of interest and/or it may be difficult to distinguish between the related proteins.

Fourthly, many receptors are expressed as multiple subtypes. Bacteriophage libraries are often incapable of distinguishing between receptor subtypes.

We have now developed procedures which overcome problems associated with known procedures.

According to one aspect of the invention, there is provided a method of producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of heterologous (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, which process comprises:

(a) subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to one or more proteins other than said hetereologous protein are bound to the cells,

(b) separating bound and unbound bacteriophage, and

(c) isolating a modified bacteriophage library depleted in library members which bind to one or more proteins other than said heterologous protein.

According to a first embodiment, the invention is especially concerned with elimination of library members which bind to native cell surface associated proteins of the selected strain. In this embodiment, in fractionation step (a), members of the bacteriophage library which bind to native cell surface associated proteins are bound to the cells, and in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to native cell surface associated proteins of the selected strain.

Thus, more specifically in accordance with the first embodiment of the present invention, there is provided a method of producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a cell surface-associated

heterologous (non-native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the cell surface-associated heterologous protein is detected, which process comprises:

(a) subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to native cell-surface associated proteins are bound to the cells,

(b) separating bound and unbound bacteriophage, and

(c) isolating a modified bacteriophage library depleted in library members which bind to native cell-surface associated proteins of the selected strain.

According to a second embodiment, the invention is especially concerned with the use of bacteriophage libraries to distinguish between closely related heterologous proteins, for example between recptors of different sub-types. Thus for many ligands, more than one receptor sub-types are known, as indicated by the following Table:

Ligand	Receptor sub-types
Neuropeptide Y	5 receptors have been currently cloned (Y1-Y5)
Tachykinins	3 receptors are known NK1-NK3
TNFαR	Two receptors are known
Noradrenalin/adrenalin	At least 10 receptors are known
Opiods	Multiple receptor types are known (probably 4)
5HT	At least 14 receptors are known
Dopamine	At least 5 receptors are known
VIP/PACAP	Three receptors are known

Thus according to this embodiment, the method of the invention is directed to the production of a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a first heterologous (non native) protein (especially a cell surface associated protein) in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the first heterologous protein is detected, wherein step (a) comprises subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said first heterologous protein, but which do express a second heterologous (non native) protein, whereby members of the bacteriophage library which bind to said second hetereologous protein are bound to the cells, and in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to said first heterologous protein.

In this method, the first and second heterologous proteins may be receptor proteins of different subtypes, whereby in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to heterologous proteins of the subtype comprising said first heterologous protein.

Thus in step (a) the initial library is contacted with cells which have been transformed so as to express the second heterologous protein (which may, for example be a sub-type of a receptor protein). Library members which bind specifically to that subtype may then be eliminated from the library and the resulting depleted library used in a subsequent detection step that is aimed at selecting library members which bind to the first heterologous protein. In this subsequent step, the depleted initial library is contacted with cells which have been transformed so as to express the second heterologous protein (which may, for example be a sub-type of a receptor protein). Bacteriophage which are selected by this step will be characterised by the ability to bind to the second heterologous protein, but not to the first. As will be appreciated, this technique is of especial use in distinguishing between receptor subtypes.

The bacteriophage library used in either embodiment would generally comprise a plurality of members possessing a random collection of oligopeptides expressed at the N-terminus of a coat protein. The oligomers may, for example be from 7 to 18 amino acids in length, specific examples being 7-mers and 15-mers.

In carrying out the method of the invention according to the first embodiment, the initial step (a) consists of fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to native cell-surface associated proteins are bound to the cells. The cells of the selected strain which do not express the heterologous protein are preferably what we refer to as "mock transfected cells", i.e. cells that have been transfected with vector which is incapable of transforming the cells to express said heterologous cell surface-associated protein.

In carrying out the method of the invention according to either embodiment, the selected strain of cells which are used to express the heterologous protein may be of diverse origins, (e.g. mammalian, avian, insect, yeast, etc.), but conveniently, any established line of cells with a well characterised associated expression system is used. Most preferably, mammalian cells, especially primate cells are used.

One particular class of cells that is convenient to use are so-called COS cells, for which a well understood transfection and expression system is available. COS cells were originally derived from African Green Monkey kidney and are stably transformed with SV40 virus. The cells express the SV40 large T antigen and are capable of effecting the transient expression of cDNA introduced in the form of an SV40-derived vector in which the cDNA is operatively linked to the SV40 origin of replication. Examples of COS cell lines are the lines COS1 and COS7, each of which is obtainable from ATTC. Other cells that may be used are murine cells such as the WOP cell line.

Vectors suitable for expressing heterologous cDNA in COS cells are described by Seed *et al.* in Nature (1987), **329**:840-842 and Proc. Natl. Acad. Sci. **84**:3365-3369 and are available from the authors thereof. One example of the vectors available from this source is the so-called CDM vector (also known as *m*H3M). Another specific vector suitable for transforming COS cells is the vector pCDM8, which is a derivative of CDM, and is available commercially from Invitrogen.

In carrying out the present invention it is advantageous to express the target protein at very high copy number. In this regard, we have taken advantage of the fact that it is possible to express proteins, especially cell surface proteins, in cell lines at high copy number and use this as our target. This approach provides a number of key advantages that allow the method of the invention to work efficiently by biasing the signal to noise for this process greatly in our favour. In the case of naturally-occurring cell surface-associated proteins, 10^5 copies/cell is

normally regarded as being a "high" copy number, 10^4 copies/cell is normally regarded as being a "medium" copy number and 1-2x10^2 copies/cell is normally regarded as being a "low" copy number. We have found that operating with copy numbers of 1-10x10^6 copies (on average) of the heterologous protein per cell is advantageous. It will be appreciated that this is an average copy number for the whole cell population which include non-transfected cells. Thus some of the cells may not be transfected at all and others may be characterised by a much higher actual copy number. At this level of expression, the heterologous protein may represent as much as 2% of the cell surface proteins.

We have found that by using the DEAE/dextran method of transformation , transfection at high copy numbers can be achieved and this can result in >30%, >50% and on occasions >65% of cells being transformed. The DEAE/dextran method of transfections is thus preferably used in carrying out the method of the invention. As will be described below, the efficacy of the method of the invention has been demonstarted by using as target, the human high affinity receptor for immunoglobulin G (IgG), expressed in COS cells.

The step defined in accordance with the invention of subjecting the initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to native cell-surface associated proteins are bound to the cells, may be termed a "pre-clear" step, which provides the important technical advantage of reducing "noise".

In more detail, by using a cell line to express the cDNA encoding a receptor, the "noise" component in the approach is reduced in accordance with the invention by employing a "pre-clear step"; thus, the bacteriophage library is first incubated with normal cells or mock transfected cells to remove from the bacteriophage library a substantial proportion, and in many instances, all those phage that bind to cell surface components that are naturally expressed, or bacteriophage that

express "sticky" peptides. By "mock transfected" is meant cells that are transfected with an "empty" vector", i.e. a vector which has not been loaded with cDNA of a heterologous protein.

Altrernatively, in the second embodiment referred to above, in the "pre-clear step", the bacteriophage library is first incubated with cells that have been transfected with said second heterologous protein to remove from the bacteriophage library a substantial proportion, and in many instances, all those phage that bind to said second heterologous protein.

The modified bacteriophage library that is recovered after the "pre-clear" steps referred to is a new product, which itself forms a further independent aspect of the present invention.

The invention further provides a modified bacteriophage library obtainable by the methods described above.

Thus the invention further provides according to the first embodiment, a modified bacteriophage library for use in conjunction with cells of a selected strain that have been transformed for expression of a heterologous (non-native) protein (especially a cell surface associated protein) in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, characterised in that the library is depleted in members that bind to one or more proteins other than said heterologous protein.

According to the second embodiment, the invention provides a modified bacteriophage library for use in conjunction with cells that have been transformed for expression of a first heterologous (non native) protein (especially a cell surface-associated protein) in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, characterised in that the library is depleted in members that

bind to a second heterologous (non native) protein (especially a cell surface-associated protein).

The modified bacteriophage libraries defined above are of commercial value as it can be amplified and used for future pannings.

Further, by increasing the signal, a "positive selection" step is provided in accordance with the invention to identify bacteriophage expressing peptides that recognise the target protein. Thus, bacteriophage from the original library that do not attach to the mock transfected or non-transfected cells (or to the cells transformed so as to express the second heterologous protein referred to in the second embodiment of the invention) are incubated with cells engineered to express the target protein at high copy number.

Thus according to a further aspect of the invention there is provided a method of screening a bacteriophage library so as to isolate or identify an individual bacteriophage of the library which displays an oligonucleotide which undergoes specific binding to a ligand recognition site of a protein (especially a cell surface-associated protein), which comprises expressing the protein by transecting a cells of a selected strain, contacting the transfected cells with the library, and selecting bacteriophage on the basis of their capacity to bind to the protein expressed by the transected a cells, characterised in that the bacteriophage library is a modified bacteriophage library as defined herein.

When operating according to this aspect of the invention the bacteriophage are preferably selected by carrying out a plurality of rounds of screening, however it is found that the number of rounds can be substantially reduced compared to prior art procedures in which the bacteriophage library has not been subjected to a "pre-clear" step in accordance with the invention.

The invention further provides a refinement of the screening procedure which allows peptides which bind specifically to ligand binding sites of the protein

(especially a cell-surface associated protein) to be identified. This refinement is of importance, because on occasions, peptide sequences in a bacteriophage library may bind specifically to a cell-surface associated protein at a location which is distal from, or not functionally associated with a ligand binding site. According to this aspect of the invention the selection procedure comprises at least one round of screening which comprises subjecting the target protein to a panning step in which the protein is contacted with the bacteriophage in the library, separating unbound bacteriophage from bacteriophage which are bound to the target protein, and subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, and recovering displaced bacteriophage.

The method of selecting from a bacteriophage library individual bacteriophage which specifically bind to a ligand receptor site of a target protein, which comprises subjecting the target protein to a panning step in which the protein is contacted with the bacteriophage in the library, separating unbound bacteriophage from bacteriophage which are bound to the target protein, and subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, and recovering displaced bacteriophage forms a further independent aspect of the invention.

Peptides identified using the procedures described herein, form further aspects of the invention, by virtue of their ability to recognise target proteins. They may thus be used commercially as therapeutic or diagnostic reagent or sequence information contained in the thus isolated or identified peptides may be used in the design of other pharmaceutical products. Options include chemical or genetic modification of the peptide or use of the native peptide itself for

diagnostics, magic bullets to deliver agents such as drugs, toxins and antibodies to cells.

As indicated, the invention provides a further selection procedure, that may be used independently from, or in combination with the step referred to as the "pre-clear" step. This further procedure involves selection of bacteriophage that express peptides that interfere with binding of the natural ligand, drugs or other factors such as antibodies that recognise the target protein.

Thus the invention further provides method of selecting from a bacteriophage library individual bacteriophage which specifically bind to a ligand receptor site of a target protein, which comprises subjecting the target protein to a panning step in which the protein is contacted with the bacteriophage in the library, separating unbound bacteriophage from bacteriophage which are bound to the target protein, and subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, and recovering displaced bacteriophage.

The procedure, which has been defined above in terms of subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, will be referred to herein as "reverse panning".

In the reverse panning step, previously selected bacteriophage (from the positive selection step) may be selected that now fail to recognise the target when the cells expressing the target are incubated with the other desired component such as the natural ligand, modified ligand, drug or antibody. In this way the

subset of bacteriophage that express proteins which bind to a specific region of the target are defined and selected.

The peptides identified by the methods of the invention (especially in the embodiments that include a "reverse panning" step) are of particular value as, or in the design of commercial products, as they can be used as ligand or drug mimetics. Information obtained from the array of peptides selected in this way can be used to define commonality of shared features and used for design of peptides with distinctive features, peptide-like structures and nonpeptide organics.

Thus the invention further provides the use of a peptide isolated or identified using the methods described herein in the manufacture of a pharmaceutical composition for treating a disease condition involving the cell surface-associated protein or its ligand.

Also, the invention further provides the use of a peptide isolated or identified using the methods described herein in the design of manufacture of a pharmaceutically active substance for treating a disease condition involving the cell surface-associated protein or its ligand.

Description of Figures

The invention will now be described in more detail, with particular reference to the accompanying drawings of which:

Figure 1 is a schematic diagram illustrating methods according to the invention,

Figures 2 and 3 are bar charts illustrating the results of selecting bacteriophage using the methods of the invention, and

Figures 4 and 5 show data generated using the BIAcore 2000 affinity analysing apparatus.

An example of an overall procedure in accordance with the invention is shown in the attached Figure 1. The example shown is expression of the DNA for FcγRI COS cells. However, other cell lines can be used throughout this process.

Pre-clear step

1. The bacteriophage library is first incubated at 4°C with COS cells that were mock transfected (i.e. cells were processed through the transfection procedure but no DNA or an irrelevant DNA was transfected into the cells). The cells and bacteriophage were kept constantly mixed at 4°C in isotonic buffer at physiological pH.

2. After allowing the bacteriophage that express peptides that recognise COS cell surface proteins to adhere to these mock transfected cells, the cells were centrifuged to sediment the cells and remove all the adherent bacteriophage. The resulting modified bacteriophage library is a new product and can be used for all subsequent manipulations. I.e. we have created a bacteriophage display library where peptide sequences that recognise COS cell surface proteins have been removed.

Positive selection

3. The supernatant from the "pre-clear" step was then incubated at 4°C with COS cells expressing the cDNA for a cell surface protein suspended in isotonic buffer at physiological pH. The cells were kept constantly mixed.

4. Bacteriophage that express peptides which recognise the target protein are allowed to attach to these transfected cells at 4°C (to prevent internalisation of the target) protein), the cells were washed repeatedly with large volumes of cold isotonic buffer at physiological pH to remove the non-adherent bacteriophage. (We found that it was desirable to wash the cells at least five times before no bacteriophage could be recovered from the wash supernatants).

5. Adherent bacteriophage were then removed by washing the cells in isotonic solution at low pH (less than pH 4). Cells were separated from the supernatant and the supernatant was neutralised.

6. The bacteriophage were then amplified in bacteria

The rounds of positive panning (steps 1 to 6; or steps 3 to 6) may be repeated, if desired.

Reverse panning

7. The natural ligand, human IgGI, was used at saturating concentrations to displace bacteriophage expressing peptides that recognise the receptor, Fc-γRI, so as to identify peptides that recognise the ligand binding pocket of the receptor.

Peptides that interact with the cell surface protein at a site close to or comprising the binding pocket for ligand can be selected through a process of reverse panning. Under these circumstances, it is necessary first to have selected the population of bacteriophage that express peptides recognising the cell surface protein by the positive selection step described above. These bacteriophage are then incubated with COS cells expressing the cell surface protein in the presence of the ligand at 4°C in isotonic buffer. The concentration and nature of the displacing molecule can be altered as necessary to select preferentially different affinity displayed peptides or peptides with different specificities. Under these circumstance, the supernatant and COS cells are separated but the bacteriophage in the supernatant are amplified as these represent bacteriophage that express target specific peptides unable to bind the cell surface protein in the presence of ligand.

8. The peptide sequence is derived using standard sequence reactions of the bacteriophage DNA

A especially valuable procedure that can be used in conjunction with the method of the invention in order to provide additional information about the binding capabilities of oligopeptide sequences identified in the initial screening involves the use of the BIAcore 2000 affinity analysing apparatus. Subsequent evaluation using this apparatus allows a rapid assessment of selected bacteriophage expressing peptides of interest.

More specifically, we have developed a rapid method of screening the binding affinities of the selected bacteriophage by using the BIAcore2000. Briefly, the bacteriophage are captured on to the chip surface using an anti-phage antibody. The cell surface-associated protein in solubilised form (in the following example Fc-γRI) is passed over the captured bacteriophage and semiquantitative binding kinetics are established. In this way, numerous bacteriophage can be assessed for variance in the on-rate and off-rate.

The following Example and the associated Figs. 2 and 3 illustrates the application of the method of the invention to isolate peptide consensus sequences that bind to the FcγRI recognition site.

EXAMPLE I

In this example, a bacteriophage library expressing 15-mer peptides was used for affinity selection with COS cells actively expressing FcγRI-MANX on the cell surface. For the derivation of the 15-mer library, see Zacher, A.N. *et al.* Gene 9, (1980) pp.127-140. FcγR1-MANX is described in Davis *et al.*, EMBO Journal, 14 (1995) pp.432-441.

1. **Generation of Transformed and Mock-Transfected COS Cells**

Transformed and mock-transfected COS cells were generated as follows:

Day 1

COS cells were split to be approximately 50% confluent the next day for transfection with FcγRI-MANX construct. 6 sterile 60mm culture dishes.

Day 2

COS cells were transected with the FcγRI-MANX construct in the presence of DEAE-DEXTRAN .

Day 3

Cells were trypsinize to remove traces of DEAE-DEXTRAN and transfer into 100mm plates as follows:

2 x transfected with FcγRI-MANX	100mm dish.
2 x mock transfected	100mm dish.

2. **Pre-Clear Step**

The "pre-clear" step was effected as follows:

Day 4

(i) To mock transfected cells (1 x 100mm dish), aspirate off cell medium, and replace with 1 x PBS to wash the cells. The COS cells were then removed from the plate with PBS-EDTA (1mM), and incubated at 37°C for 10 minutes. Complete removal of cells was checked by microscopy.

(ii) Cells were lifted off with a sterile 10ml pipette and transferred to a sterile 15ml T.C. tube for sedimenting at 1,000xg; 5 minutes at 4°C. The cell pellet was resuspended in cold PBS-BSA 1% (1ml) and kept on ice.

(iii) 30μl of the 15-mer library (stock 4.4 x 10^{11} phage/ml) was taken and the phage were diluted in 1ml of PBS-BSA before mixing with the mock-transfected COS cells. The mock-transfected COS cells were contacted with the bacteriophage library and incubated at 4°C for 1 hour with gentle rotation.

(iv) For titring, a culture set up of K91 cells (5ml) inoculated from a single colony. Mid-log phase reached $\approx$ 4 hours. K91 cells were also plated onto minimal media to maintain F' and streaked onto Kan/LB plates. Kanamycin (50μg ml^{-1}).

(v) Mock transfected cells were removed from the cold room after 1hr and centrifuged at 1,500xg for 5 minutes at 4°C. Samples of the supernatant and pellet fractions were retained for titre determination.

MTC P1 (pellet) dil. range 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}.

MTC S1 (supernatant) dil. range 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.

3. Panning

(vi) COS cells expressing FcγRI-MANX constuct were lifted off as described in section (i) above, resuspended in 1ml of cold PBS-BSH and mixed with 2mls of the supernatant from step (v). The mixed suspension [COS/FcγRI-MANX and phage] were incubated at 4°C for 1hr with gentle rotation.

(vii) [COS cells/ FcγRI-MANX and phage] suspension was removed from the cold room after 1hr. The suspension was layered over a 2% FICOLL cushion (prepared in PBS/BSA) kept on ice i.e. 3mls of suspension layered over a 3ml 2% FICOLL cushion. Pellet cells and binding phage for 5 minutes, 1,500xg at 4°C.

(viii) COS cells which pellet contain phage specific and non-specific for the MANX receptor. The supernatant was carefully decanted and a sample retained for titring.

(ix) The pellet was washed 1x with 10ml PBS/BSA 1% and re-spun 1,500xg for 5 minutes at 4°C. The wash was saved for titre determinations.

(x) The resultant pellet was washed with acidified PBS (pH 2-5) 1ml for 5 minutes at 4°C. Cells were pelleted at 1,500xg for 5 minutes at 4°C. The supernatant was transferred to a sterile Ependorf tube and neutralized with 150μl of 1M Tris HCl, pH 9.0. A sample of the eluate was retained for titre determination as follows.

CFM S1 = 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} ,

CFM W1 = 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},

CFM E1 = 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},

The results of the first round pannning are shown in Fig.3.

4. Third Round Screen of 7-mer Peptide Library

Day 1

Cos cells (60mm) were split into 6 x 60mm plates to about 50% confluent, next day for transfections.

Day 2

FcγRI-MANX DNA was prepared for transfection, 5μl of DNA in 200μl of TE (one Ependorf for each transfection; 3 were set up). The DNA was diluted in TE as above and 200μl of DEAE-DEXTRAN was added. The solution was left to stand at room temperature for 30 minutes. In the mean time, media

aspirated off COS cells and replaced with 1.5ml of NU medium plus the addition of 1.5μl of chloroquine. The cells were left at 37°C for 1 minute. Media was removed from the plates and replaced with 2mls of warm NU media (37°C). The DNA mix was then added drop by drop and the cells incubated at 37°C for up to 4h.

After 4h, the cells were shocked ie. NU medium was removed and replaced with 5ml of 10% DMSO/PBS and incubated for 2 minutes. DMSO/PBS was replaced with 1 x PBS and aspirated off. Fresh DMEM medium was added and the cells incubated at 37°C overnight.

5. Amplification

The peptide library was amplified. 1ml of the library was added to 20ml of ER2537 culture at mid-log point and grown at 37°C with vigorous shaking for 48 hours. The culture was transferred to a Falcon tube and spun at 3,000rpm in a Beckman GS-6KK centrifuge (4°C) for 15 minutes to pellet bacterial cells. The supernatant was transferred to a fresh tube and respun. 80% of the supernatant was transferred to a fresh tube and precipitated phage allowed to form overnight at 4°C (after addition of 1/6vol. PEG/NaCl).

The phage-library was cleaned up and titred. The PEG precipitated phage were pelleted for 15 minutes at 10,000rpm at 4°C. The supernatant was decanted, respun once and the residual supernatant removed with a pipette.

The pellet (phage) was suspended in 1ml of sterile PBS. The suspension was transferred to a centrifuge tube and spun at 13,000rpm - Beckman Cold Room, 5 minutes to pellet residual cells.

The supernatant was then transferred to a fresh 1.5ml Ependorf tube and precipitated with 1/6th vol PEG/NaCl and incubated on ice for one hour. The precipitate was pelleted at 4°C, 13,000 Beckman centrifuge. The supernatant was

discarded and the pellet suspended in 1ml of PBS pH 7.4. The supernatant amplified was stored at -20°C after the addition of DMSO to 7% final volume = 70μl.

The amplified eluate was titred for second round screen. A single colony from a minimum medium plate for strain ER2537 was picked and used to inoclate 5ml of LB. The E.coli were allowed to grow for 3 hours until they reached mid-log phase (OD 600~0.5-1.0. Phage were diluted N-10^{12}.

3mls of molten agar was displaced into 12ml sterile tubes for each dilution and incubated at 50°C until required.

10μl of each dilution (phage) were incubated for 5-15 mininutes at room temperature with 200μl of mid-log ER2537 cells. The suspension (phage-bacteria) were added to each tube and mixed (gentle vortex) and poured onto LB agar plates previously dried and equilibrated to 37°C.

Once the LB agar set, the plates were incubated overnight at 37°C (inverted) in ovens without a fan.

Day 3

COS cells were trypsinized and plated onto fresh culture dishes, supplemented with fresh DMEM and incubated at 37°C.

The phage (amplified) were titred.

6. Pre-clear

Day 4

72h. COS cells after transfection.

Mock transfected COS cells (MTC) were washed with PBS, 2x to remove traces of DMEM media and dead COS cells. The cells were removed from the surface with PBS/2mM EDTA (2μl) per 60mm culture dish. This was enhanced by incubation for 10 minutes at 37°C. Removal of cells from the surface was checked by microscope. The cells were pelleted 1,000rpm x 5 min. at 4°C. The cells were resuspended in PBS:BSA (1%), (10ml) and washed 1x by gentle inversion on ice. The cells were pelleted by centrifugation as before. The resultant pellet was resuspended gently in 500μl of PBS/BSH cold (4°C).

To the 500μl suspension (MTC), 1ml of the phage suspension in PBS was added. The mixture was incubated at 4°C with gentle rotation for 1h. After incubation the phage/MTC suspension was centrifuged at 1000rpm 4°C for 5 min. Fractions from MTC-S1 (supernatant) and MTC-P) were saved for titre.

7. Panning

The MTC-S1 (1.49mls) was obtained. Transfected cells with FcγRI-MANX (CFM) were removed from the 60mm dishes (x3 as described above for MTC cells). The CFM cells were suspended in 500μl of PBS-BSA 4°C and MTC-S1 (1.490mls) was added. The resultant mix was gently rotated for 1h at 4°C.

After rotation, the CFM/Phage mix was centrifuged at 1,000rpm for 5 minutes at 4°C. The CFM-S1 (supernatant) was carefully removed and retained

for titring. The CFM pellet/phage was washed 5x (W1-W5) with 10mls of PBS-BSA (4°C). The washings were performed on ice, by gentle inversion spinning down between washes.

The pellet CFM after the final wash was suspended in 1ml of acidified PBS pH2.5 fora 5 min at 4°C. The solution was mixed gently by inversion. The CFM/PBS 2.5 was centrifuged at 1,000rpm x 5 minutes at 4°C to pellet the COS cells. The CFM-P1 and CFM-E1 were retained for titring. The eluate ≈1ml was neutralised with 250µl of 1MTris-HCl pH 9.2.

8. Negative Panning of 7-mer and 15-mer

Day 1

COS cells (8 x 60mm plates) were split into 6 x 100ml plates to be approx 50% confluent by next day. Each 17-mer and 15-mer peptide library was amplified.

1ml of each eluted fraction (for each library) was added to either 20ml of ER2537 culture (7-mer) or K91 cells (15-mer) at mid-log phase and grown at 37°C with vigorous shaking for between 4-5 hours Each culture was transferred to a Falcon tube and spun at 3000 rpm in a Beckman GS-6KK centrifuge (4°C) for 15 minutes to pellet the bacterial cells. The supernatant was transferred to a fresh tube and respun. 80% of the supernatant was transferred to a fresh tube and phage allowed to precipitate overnight at 4°C after the addition of 1/6 vol PEG/NaCl.

Day 2

FcγRI-MANX DNA was prepared for transfections 5µl of DNA in 200µl TE (1 Ependorf per each transfection (4 set up)).The DNA was eluted in TE as above and 200 µl of DEAE Dextran was added. The solution was left to

stand at room temperature for 20 minutes . In the meantime, media was aspirated off the COS cells and replaced with 1.5 ml of NU medium plus the addition of 1.5 ml of chloroquine. Included were COS cells mock-transfected with the above excluding DNA. The cells were left at 37°C for 1 minute . Media was removed from the plates and replaced with 2ml of warm NU medium (37°C). The DNA mix was then added drop by drop and the cells incubated at 37°C for up to 4 hours.

After 4 hours the cells were DMSO/PBS shocked - NU medium was removed and replaced with 10% DMSO/PBS solution. and incubated for 2 minutes. DMSO/PBS was replaced with 1xPBS to wash the cells and replaced with fresh DMEM medium. The cells were incubated overnight at 37°C

The phage libraries were cleaned up (filtered 4.5μm filter) see p 40.

Day 3

COS cells were trypsinised and plated with fresh culture medium supplemented with fresh DMEM and incubated at 37°C . Amplified phage from the day before were titred.

Day 4

72 hr COS cells after transfection . Mock transfected COS cells were washed with PBS 2x to remove traces of DMEM media and dead COS cells. The cells were removed from the Petri surface with PBS 2mM EDTA 5ml). per 100mm plate. This was enhanced by incubation for 10 minutes at 37°C. Removal of cells from the surface was checked by microscopy. COS cells were pelleted 1000 rpm for 5 minutes at 4°C. The cells were resuspended in PBS BSA (1%) (10mls) and washed 1x by gentle inversion on ice. The

cells were pelleted by centrifugation as before. The resultant pellet was resuspended gently at 4°C in 500 µl of PBS/BSA (cold 4°C).

To the 500µl suspension (MTC) 1ml of the phage suspension in PBS was added . Separate tubes for each library . Each library phage suspension was incubated at 4°C with gentle rotation for 1 hr

After incubation, the phage/MTC suspension was centrifuged at 1000 rpm at 4°C for 15 minutes . Fractions from the MTC-P1 and MTC-S1 were saved for titring.

Parallel to the above, COS cells transfected with cγRI-MANX DNA (CFM) were incubated near the end IgG1 (human) 500µl of CFM cells with PBS-BSA - 5:100 dilution of human IgGI (3.3x10-7) saturation, 4°C all incubations were carried out in the cold room on ice at 4°C.

The MTC-s1 fraction for each library (~1.5ml) was added to the 500 µl of COS cells (CFM) preincubated with IgG1 and rotated gently at 4°C for 1 hr.

The COS cells (CFM) were pelleted by centrifugation as before and the supernatant retained for titering. The phage blocked in their binding to FcγRI. To test for a lack of internalisation, The COS cells in the pelleted fraction were goat anti-human Igg1 FITC-labelled and checked by microscopy-. Result produced the ring structure around cells (labelling normally produced suggesting no receptor internalisation)

The rsults of the reverse panning procedures are shown in Fig. 2

MEDIUM DEFINITIONS

DMEM	Dulbecco's Modified Eagles Medium
NU	DMEM plus 10% NU (Newborn calf) serum
PBS	Phospate buffered saline (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO4, 0.24g of KH_2PO_4, add distilled water to a total volume of 1 litre. Adjust pH to 7.4 with NaOH and autoclave.
PBS/BSA	PBS plus 1% (w/v) BSA
PBS/DMSO	PBS plus 10% (v/v) DMSO
PEG/NaCl	To 200g of Polyethylene glycol 6000 and 146.1g of NaCl, add distilled water to 1 litre, heat to dissolve and autoclave.
LB	10g/l bactotryptone; 5g/l bacto-yeast extract; 10g/l NaCl; and adjust pH to 7.0 with NaOH. For solid media, 15g/l agar was added before autoclaving. After autoclaving (121°C, 15p.s.i.), and the media was cooled to 55°C before antibiotics were added.
T.E.	10Mm tRIS-hcL, Ph7.4, 1Mm EDTA.

10. Identification and Sequences of Isolated Phage

After three rounds of positive enrichment/selection and one round of reverse panning, bacteriophage having peptides with the following sequences were identified (multiple sequence alignment of individual 15-mer peptide sequences are shown):

TABLE

```
K16  - - - - Q P P K Q K A P A M P G H S T - - - - - - - - - -
K30  - - P L K L I G Q P S T A L P P H - - - - - - - - - - - -
K15  - - - L L V L A P P A A A F S Q M G - - - - - - - - - - -
K19  - - - - - - - I P R L S A Q P P F P Q W T I - - - - - - -
K24  W T S G P A L G R C S T O V P - - - - - - - - - - - - - -
K2   - - - - - R K Y Q P A S R S E R P L S S - - - - - - - - -
K3   - - - - - W V R Q T S A R K A R H L S S - - - - - - - - -
K23  - T E L R G V E T F P A R S P R - - - - - - - - - - - - -
K13  - - - - - - - - - - - A Q A W C H Q S C D E T H P A - - -
K27  - - - - - - - - - D F A Q A       Q D H D Q T S Q R M - -
K7   - - - - - - - - G M P S I S M S A L T A S Y S - - - - - -
K9   - - - - - - - - - L G T L   E R V I T T S W S T G - - - -
K28  - - - - - - - - Q T N S L     R L M T H P R L T S - - - -
K1   - - - - W R M A T M P I       L L W P G P W - - - - - -
K5   - - - - - - - - - L Q P W     L L L P P P Q S V W V - -
K29  - - M T S T M P V S S V       M L L W N - - - - - - - - -
K18  - - - - L H S G M M P       V G L A I M R L - - - - - - -
K20  - - - - R P K S L M P       M A L A Q P A - - - - - - - -
K14  - - - - V L A V M N P       N T L S I Q L S - - - - - - -
K21  - - - - - P P L I E P L     H F L P Y Q L M - - - - - - -
K25  - H P F P Q L L A V A   G S S Q S - - - - - - - - - - - -
K6   - - P P E Q Y F P L A K A H S Q P - - - - - - - - - - - -
K12  - - - - T D P R A W P       S Q L Y I P G W - - - - - - -
K26  - - - - - - - - - - Y P     P Q L C P P F P L L P A P - -
K17  - - - - - - - - A G T P     P T S L H Q P K L S - - - - -
K4   - - - - - Q T V I R A     P S A F Y E P R R - - - - - - -
K8   - - - - - - - - A R T S     P D H N D P Q P I P L - - - -
K10  - T Q N R S P Y F A W H D V T M - - - - - - - - - - - - -
K11  - - - - Q Y Q Y V P I Q L R W L L P - - - - - - - - - - -
K22  - - - - - - - - - - - - - - - - - - - - - - - - - - - N V

Consensus                P   A P   L L   P
```

Following further analysis of the recovered bacteriophage using the BIAcore 2000 affinity analysing apparatus (see Figures 4 and 5 for data generated), phage K1, K5, K25 abd K26 were selected as having specific relatedness to the IgG1 binding site of the FcγRI protein.

Multiple sequence alignment of these four 15 mer peptides containing the consensus motif (LL flanked by P on either side) to the lower hinge region of human IgG1 is given as follows:

```
IgG1   E P K S C D K T H T C P P C P A P E L L G G P
K26              Y P P Q L C P P F P       L L P A P
K25                        H P F P     Q L L A V A G S S Q S
K5                           L Q P   W L L L P P P Q S V W V
K1                     W R N A T M P   I L L W P G P W

Consensus                    P  P      L L   P
```

EXAMPLE II

Using the procedures set forth in Example I above, the following ligands were identified that bind to the TNF-α and to the neuropeptide Y-Y1 receptor (NPY-Y1 receptor):

II(a) - 7-mers that bind to the TNF-α receptor

SQYFPVH
SQYSPPL
STLTQYS
YSQKAHV

II(B) - 15-mers that bind to the TNF-*α* receptor

HLAVSLALCPPLLQC
HYPYLREIYTANASC
TFPDQAWPPQFPHPY

II(C) - 7-mers that bind to the NPY-Y1 receptor

VSLNRTQ
ALRHPPP
TMSDPHS

The ALRHPPP peptide qequence was first observed in round 4 of panning and became increasingly predominant in subsequent rounds of panning, to be the only sequence found in the lasr round, cycle 7. The TMSDPHS peptide sequence was observed with increasing frequency in rounds 4, 5 and 6 of biopanning

CLAIMS

1. A method of producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a heterologous (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, which process comprises:

(a) subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the bacteriophage library which bind to one or more proteins other than said hetereologous protein are bound to the cells,

(b) separating bound and unbound bacteriophage, and

(c) isolating a modified bacteriophage library depleted in library members which bind to one or more proteins other than said heterologous protein.

2. A method according to Claim 1 in which in fractionation step (a), members of the bacteriophage library which bind to native cell surface associated proteins are bound to the cells, and in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to native cell surface associated proteins of the selected strain.

3. A method of producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a cell surface-associated heterologous (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the cell surface associated heterologous protein is detected, which process comprises:

(a) subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said heterologous protein, whereby members of the

bacteriophage library which bind to native cell surface associated proteins are bound to the cells,

(b) separating bound and unbound bacteriophage, and

(c) isolating a modified bacteriophage library depleted in library members which bind to native cell surface associated proteins of the selected strain.

4. A method according to Claim1 of producing a modified bacteriophage library suitable for use in combination with cells of a selected strain that have been transformed for expression of a first (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the first heterologous protein is detected, wherein step (a) comprises subjecting an initial library containing said individual bacteriophage and other bacteriophage to a fractionation procedure which comprises contacting the initial bacteriophage library with cells of said selected strain which do not express said first heterologous protein, but which do express a second heterologous (non native) protein, whereby members of the bacteriophage library which bind to said second hetereologous protein are bound to the cells, and in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to said first heterologous protein.

5. A method according to Claim 4 wherein said first and second heterologous proteins are receptor proteins of different subtypes, whereby in step (c) a modified bacteriophage library is isolated which is depleted in library members which bind to heterologous proteins of the subtype comprising said second heterologous protein.

6. A method according to Claim 5, wherein the first and second heterologous proteins are different receptor subtypes that recognise a common ligand, whereby the depleted library is capable of being used to distinguish between said subtypes.

7. A method according to any preceding claim in which the bacteriophage are filamentous coli phage.

8. A method according to Claim 7 in which the bacteriophage are coli phage M13.

9. A method according to any preceding claim in which the bacteriophage library comprises a plurality of members possessing a random collection of oligopeptides expressed at the N terminus of a coat protein.

10. A method according to Claim 9 in which the oligomers are from 7 to 18 amino acids in length.

11. A method according to Claim 10 in which the oligomers are 7 amino acids in length.

12. A method according to Claim 11 in which the oligomers are 15 amino acids in length.

13. A method according to any preceding claim in which the selected cells are mammalian cells.

14. A method according to Claim 13 in which the selected cells are primate cells.

15. A method according to Claim 14 in which the selected cells are COS cells.

16. A method according to any preceding claim in which the cells of the selected strain which do not express the heterologous protein are mock transfected cells that have been transfected with vector which is incapable of transforming the cells to express said heterologous protein.

17. A method according to Claim 16 in which the cells of the selected strain which do not express the heterologous protein are mock transfected cells that have been transfected with vector which is incapable of transforming the cells to express any heterologous protein.

18. A modified bacteriophage library obtainable by the method of any preceding claim.

19. A modified bacteriophage library for use in conjunction with cells of a selected strain that have been transformed for expression of a heterologous (non native) in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, characterised in that the library is depleted in members that bind to one or more proteins other than said heterologous protein.

20. A modified bacteriophage library for use in conjunction with cells of a selected strain that have been transformed for expression of a heterologous (non native) cell surface-associatedprotein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the cell surface associated heterologous protein is detected, characterised in that the library is depleted in members that bind to native cell surface associated proteins of the selected strain.

21. A modified bacteriophage library for use in conjunction with cells that have been transformed for expression of a first heterologous (non native) protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the heterologous protein is detected, characterised in that the library is depleted in members that bind to a second heterologous (non native) protein.

22. A modified bacteriophage library for use in conjunction with cells that have been transformed for expression of a first heterologous (non native) cell surface-associated protein in a screening procedure in which specific binding between an individual bacteriophage of the library and a recognition site of the cell surface associated heterologous protein is detected, characterised in that the library is depleted in members that bind to a second heterologous (non native) cell surface-associated protein.

23. A method of screening a bacteriophage library so as to isolate or identify an individual bacteriophage of the library which displays an oligonucleotide which undergoes specific binding to a ligand recognition site of a protein, which comprises expressing the protein by transecting a cells of a selected strain, contacting the transfected cells with the library, and selecting bacteriophage on the basis of their capacity to bind to the protein expressed by the transected a cells, characterised in that the bacteriophage library is a modified bacteriophage library as claimed in any of Claims 18 to 22.

24. A method of screening a bacteriophage library so as to isolate or identify an individual bacteriophage of the library which displays an oligonucleotide which undergoes specific binding to a ligand recognition site of a cell surface associated protein, which comprises expressing the cell surface associated protein by transecting a cells of a selected strain, contacting the transfected cells with the library, and selecting bacteriophage on the basis of their capacity to bind to the cell surface associated protein expressed by the transected a cells, characterised in that the bacteriophage library is a modified bacteriophage library as claimed in any of Claims 18 to 22.

25. A method according to Claim 24 in which the bacteriophage are selected by carrying out a plurality of rounds of screening.

26. A method according to any of Claims 23 to 25 in which the selection procedure comprises at least one screening which comprises subjecting the target protein to a panning step in which the protein is contacted with the bacteriophage in the library, separating unbound bacteriophage from bacteriophage which are bound to the target protein, and subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, and recovering displaced bacteriophage.

27. A method of selecting from a bacteriophage library individual bacteriophage which specifically bind to a ligand receptor site of a target protein, which comprises subjecting the target protein to a panning step in which the protein is contacted with the bacteriophage in the library, separating unbound bacteriophage from bacteriophage which are bound to the target protein, and subjecting bacteriophage which are bound to the target protein to a displacement step which comprises contacting bacteriophage which are bound to the target protein with the ligand that corresponds to the ligand receptor site, whereby bacteriophage that are bound at the ligand receptor site are displaced, and recovering displaced bacteriophage.

28. A peptide identified by the screening procedure claimed in any of Claims 23 to 27.

29. The use of a peptide as claimed in Claim 28 in the manufacture of a pharmaceutical composition for treating a disease condition involving the protein or its ligand.

29. The use according to Claim 29, wherein the protein is a cell surface associated protein.

30. The use of the amino acid sequence of a peptide as claimed in Claim 29 in the design of manufacture of a pharmaceutically active substance for treating a disease condition involving the protein or its ligand.

31. The use according to Claim 30, wherein the protein is a cell surface associated protein.

FIG. 1 1/7

SCHEME FOR BACTERIOPHAGE PANNING AGAINST COS CELLS

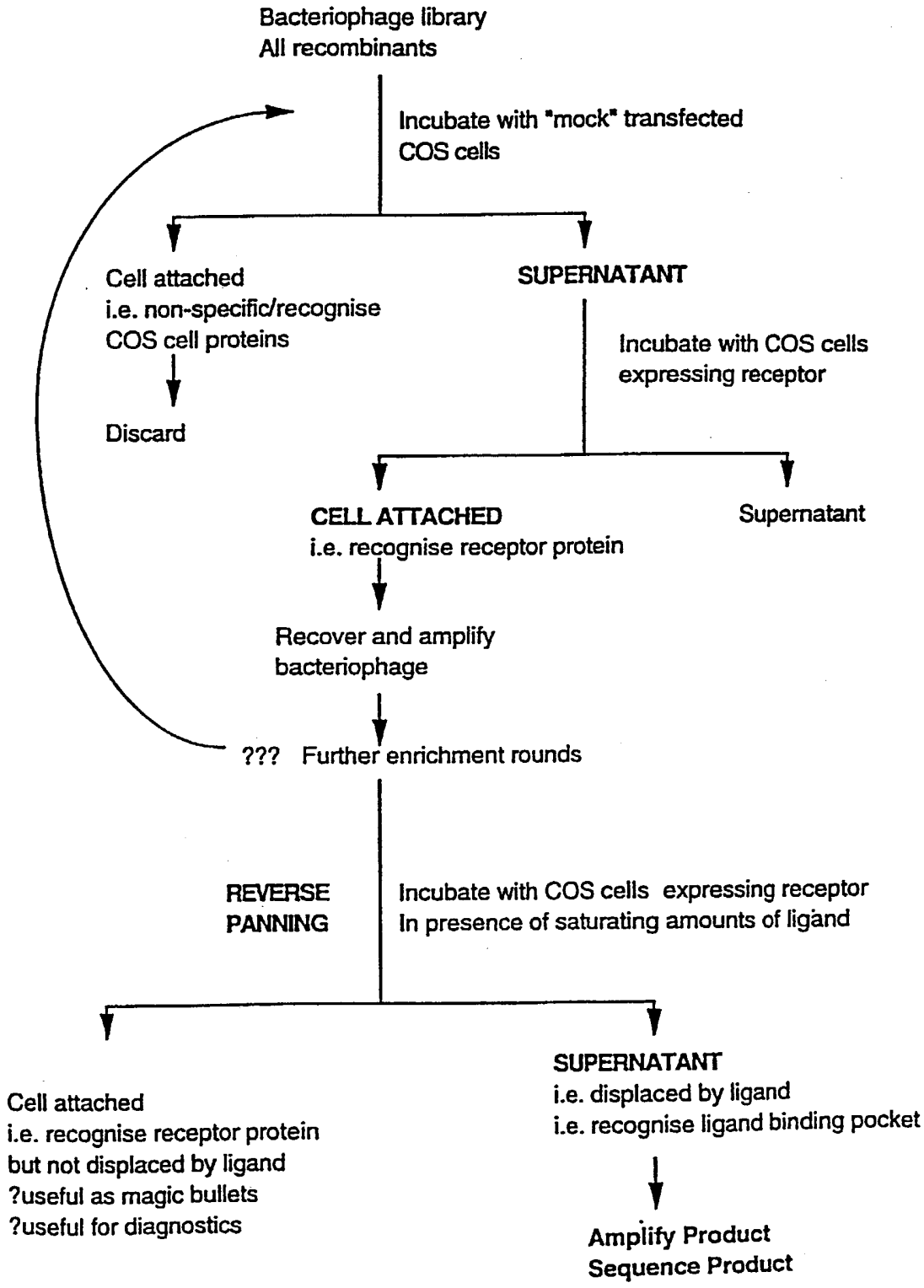


FIG. 2 Reverse panning with 15-mer and 7-mer FcγRI-specific phage against COS cells expressing FcγRI in the presence of saturating conditions of human ^{4}IgG1.

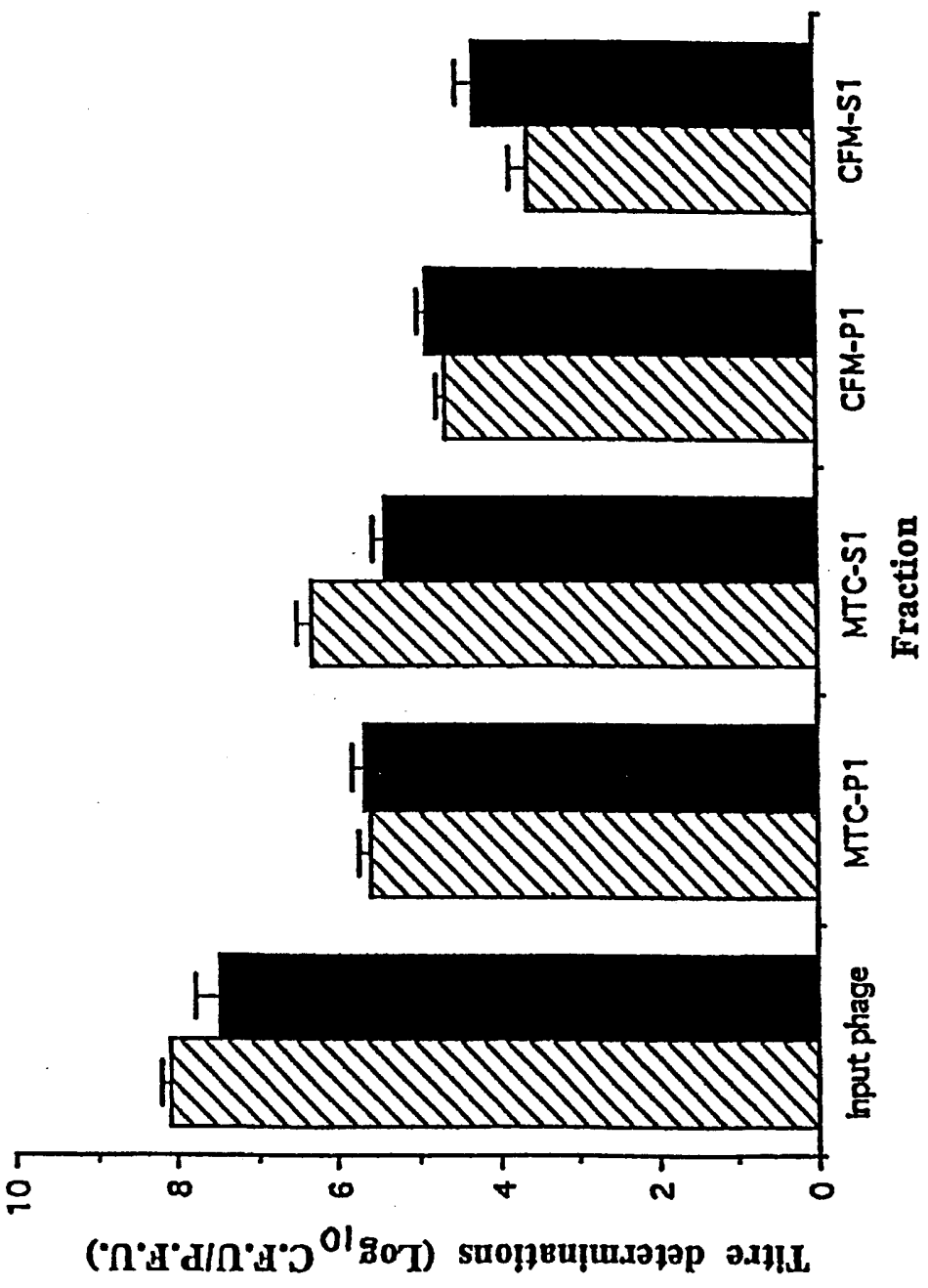


FIG. 3 First round panning with 15-mer library against FcRI

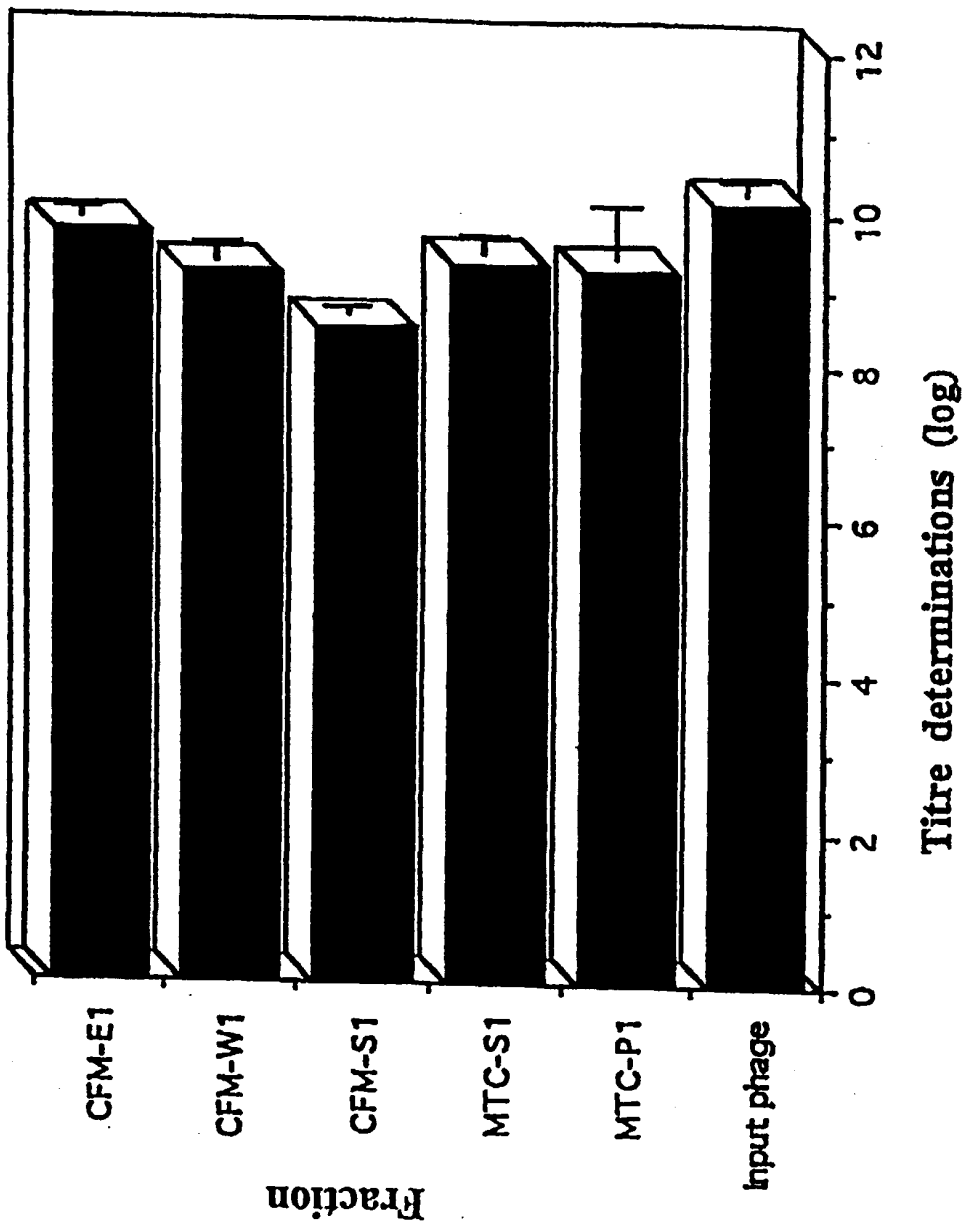


FIG. 4 Data from "15-mer on/off 8/5/97"

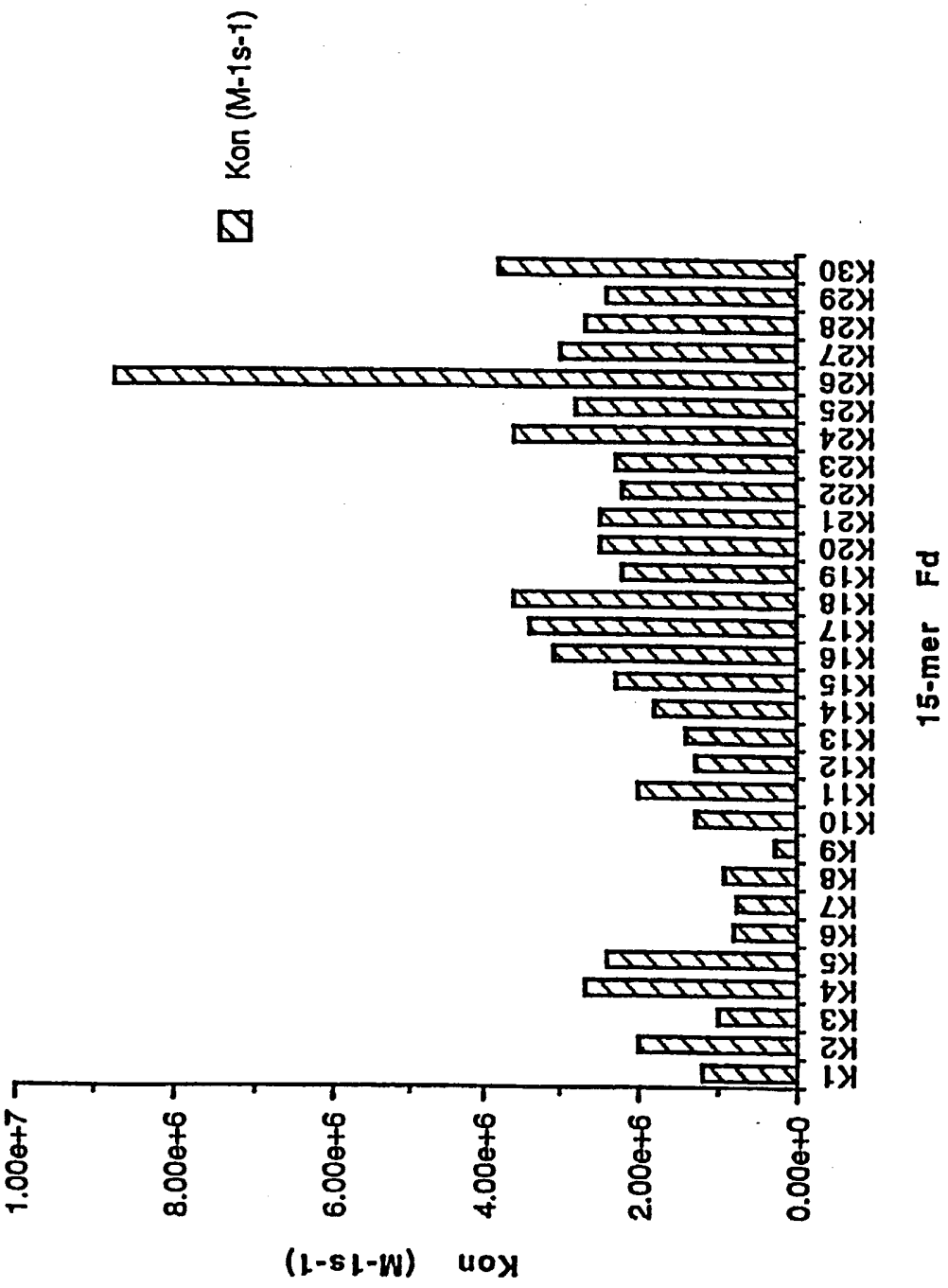


FIG. 4 (contd.) Data from "15-mer on/off 8/5/97"

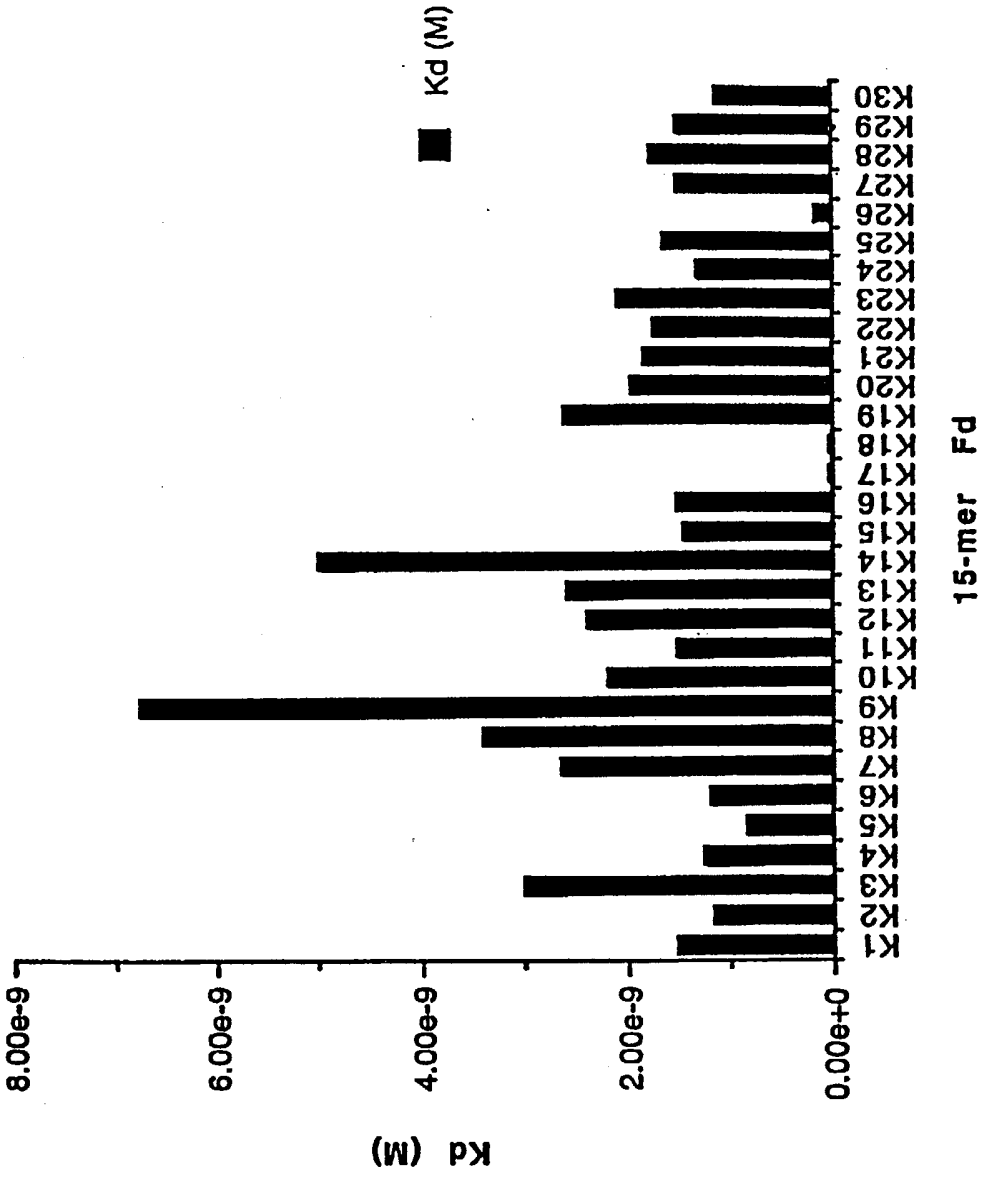


FIG. 5 Data from "15-mer on/off 8/5/97"

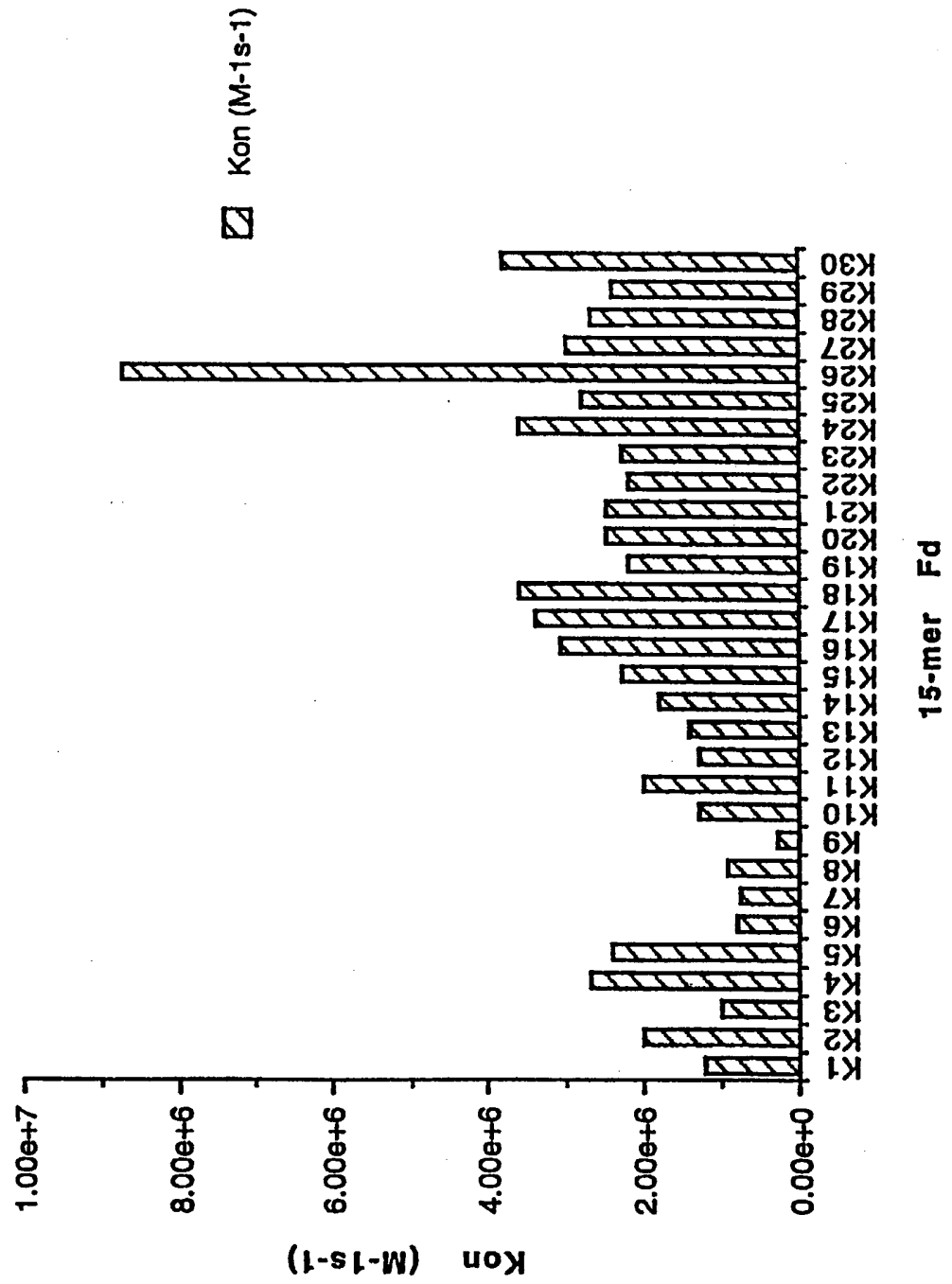
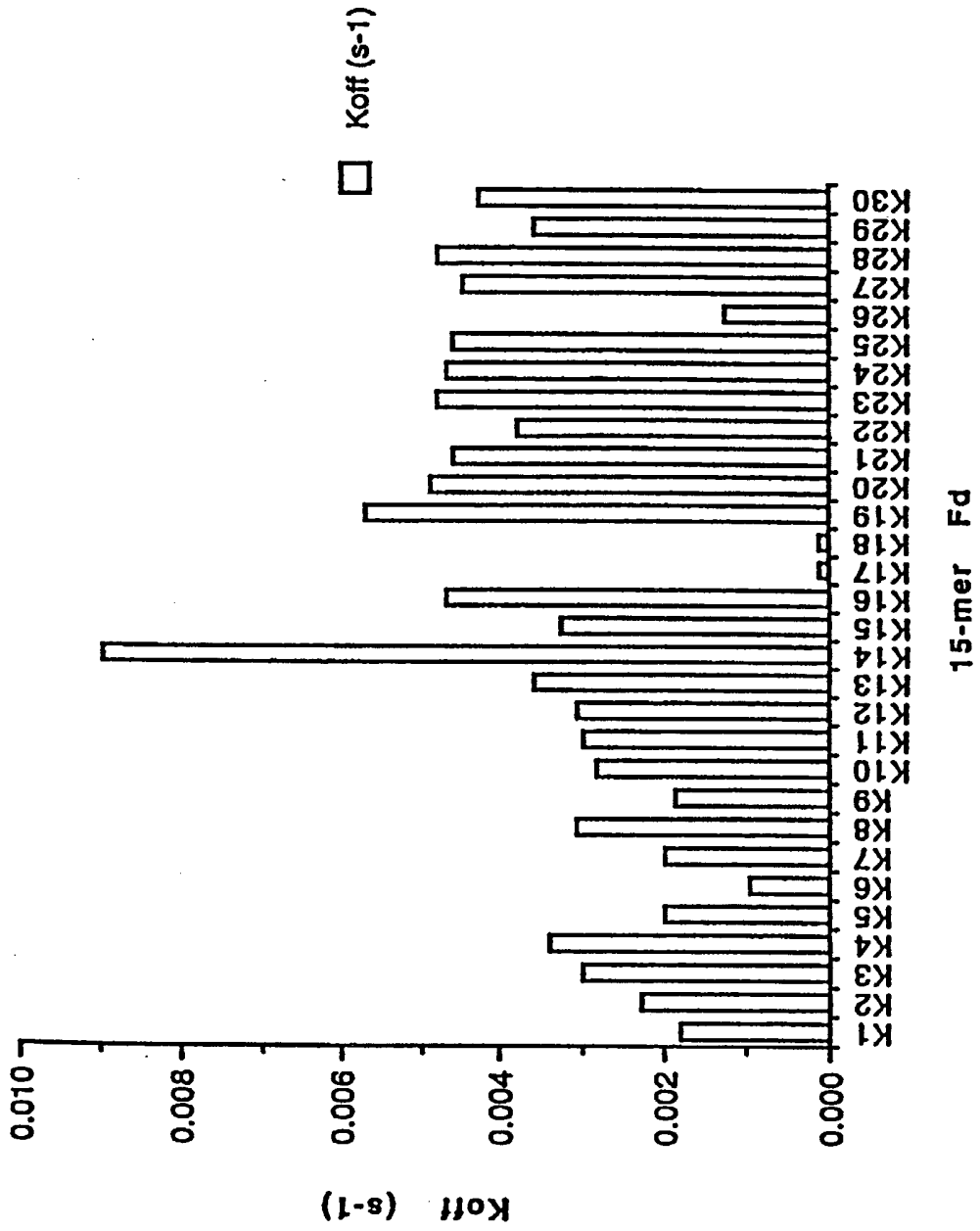


FIG. 5 (contd.) Data from "15-mer on/off 8/5/97"



INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/GB 98/02269

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 15702 A (A R BRADBURY) 17 September 1992 cited in the application see the whole document ---	18-31
A	P TSUI ET AL. : "Isolation of a neutralizing human RSV antibody from a dominant, non-neutralizing immune repertoire by epitope-blocked panning" JOURNAL OF IMMUNOLOGY, vol. 157, no. 2, 1996, pages 772-780, XP002088282 BALTIMORE US see the whole document --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1998	12/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

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INTERNATIONAL SEARCH REPORT

In..ational Application No
PCT/GB 98/02269

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	R S AMES ET AL.: "Isolation of neutralizing anti-C5a monoclonal antibodies from a filamentous phage monovalent Fab display library" JOURNAL OF IMMUNOLOGY, vol. 152, no. 9, 1994, pages 4572-4580, XP002088283 BALTIMORE US see the whole document	1-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ..ational Application No
PCT/GB 98/02269

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9215702 A	17-09-1992	EP 0575410 A	29-12-1993